The role of viral and immunological factors in congenital cytomegalovirus infections

Ph.D. Thesis

Angéla Lukácsi M. Sc.

Department of Medical Microbiology and Immunobiology

University of Szeged
Faculty of Medicine

Szeged

2005

Contents

Publications with results incorporated in the thesis

I. **A. Lukácsi**, B. Taródi, E. Endreffy, Á. Bábinszki, A. Pál, R. Pusztai: Human Cytomegalovirus gB Genotype 1 Is Dominant in Congenital Infections in South Hungary. Journal of Medical Virology 65: 537-542 (2001). I.F.: 2.881

II. R. Pusztai, **A. Lukácsi**, I. Kovács: Mother-to-fetus Transmission of Cytomegalovirus (a review). Acta Microbiologica et Immunologica Hungarica 51: 385-401 (2004). I.F.: 0

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Final Program, Abstracts 66

V. **A. Lukácsi**: Genotyping of Human Cytomegalovirus Strains Associated with Congenital Infections. Abstract International School for Molecular Biology and Microbiology (ISMBM), November 13-18, 1999, Smolenice, Slovakia. P-37

VI. **A. Lukácsi**, B. Taródi, Á. Bábinszki, A. Pál, R. Pusztai: Association of Cytomegalovirus Glycoprotein B Genotype with Congenital Infections. 7th Annual Meeting of the International Herpes Management Forum, December 3-5, 1999, Seville, Spain

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XI. **A. Lukácsi**, B. Taródi, Á. Bábinszki, A. Pál, R. Pusztai: Human Cytomegalovirus gB Genotype 1 Is Predominant in Congenital Infection. Abstract 6th Congress of Alps-Adria Society of Immunology of Reproduction, June 27-30, 2000, Pécs, Hungary. P10

XII. L. Rojkó, Á. Bábinszki, **A. Lukácsi**, A. Pál, R. Pusztai: Strain-specific Neutralization of Human Cytomegalovirus by Sera of Pregnant Women. Abstract 6th Congress of Alps-Adria Society of Immunology of Reproduction, June 27-30, 2000, Pécs, Hungary. P11

XIII. Pusztai R., **Lukácsi A.**, Nagy A., Taródi B., Endreffy E., Rojkó L., Szekeres É., Töröcsik K., Bábinszki Á., Pál A., Streitman K., Pintér S.: Virális és immunológiai tényezők szerepe a congenitális cytomegalovírus fertőzés patogenezisében. Magyar Orvosok Milleniumi Világtalálkozója, 2000. augusztus 16-19., Szeged, Magyarország Összefoglalók 108. 97. oldal

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Final Program and Abstracts, P5-27: 156

Abbreviations

AI	avidity index
AIDS	acquired immunodeficiency syndrome
CPE	cytopathic effect
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
GE	genome equivalent
HCMV	human cytomegalovirus
HIV	human immundeficiency virus
IgG	immunglobulin G
IgM	immunglobulin M
IUGR	intrauterine growth retardation
OD	optical density
PCR	polymerase chain reaction
PFU	plaque forming unit
RFLP	restriction fragment length polymorphism

1. Summary

Congenital human cytomegalovirus (HCMV) infection is the leading infectious cause of mental retardation, sensorineural deafness and visual impairment. It is mainly related to a primary maternal infection. The placenta should be considered the most important site of both the protection of the fetus from HCMV infection and the transmission of HCMV from mother to fetus.

The transmission of human cytomegalovirus from mother to fetus is a conundrum. Neither virologic nor host factors have been identified that are predictive of vertical transmission in a given mother-infant pair. It has been shown that healthy HCMV seropositive subjects may be simultaneously infected with different HCMV glycoprotein B (gB) genotypes. Since little is known about the epidemiology of different HCMV strains in maternal to fetal transmission, we characterized the HCMV from maternal, fetal and newborn specimens and placentae, and studied the impact of these strains on the clinical outcome of the infection. Genotyping on HCMV from congenital infections was carried out on the assumption that the envelope gB may influence the outcome of prenatal infection. Sixty-three pregnant women were included in the study: 40 pregnant women whose fetuses were strongly suspected of having viral infection, and 23 women with normal pregnancies, from whom amniotic fluid was taken for fetal karyotype assessment. The amniotic fluid, placenta, maternal and fetal peripheral leukocytes, serum and urine of the newborns were examined for HCMV DNA by a nested polymerase chain reaction (nPCR), and the gB genotype was determined by restriction fragment length polymorphism (RFLP). HCMV DNA was detected in 12 cases in which the fetuses were suspected of having a viral infection and in 3 of the normal pregnancies. All the HCMV DNAs had identical genotype, gB1. In the matched samples from mother-infant pairs the genotype of HCMV was similar. None of the mothers were infected with more than one gB genotype. The clinical outcomes of the congenital infections varied. Our data indicate a predominance of the gB1 genotype in congenital infection, which could reflect the infection type of the study population. The clinical outcome of these pregnancies, however, cannot be predicted on the basis of the involvement of this genotype.

Seroimmunity to human cytomegalovirus prior to conception provides substantial protection against symptomatic infection of a newborn. The most important parameter for neutralization of HCMV is the titer of neutralizing antibodies. The major target for them on HCMV is the envelope glycoprotein B. The present study was undertaken to find out if pregnant women and their matched controls differ with regards to the prevalence of IgG and neutralizing antibodies specific to Towne (gB type 1) and to AD169 (gB type 2) strains of HCMV. Furthermore we measured the capacity of neutralizing antibodies in the serum or in the plasma by a microneutralization assay. The study group consisted of 90 women (age 18-45 years); 30 women with normal pregnancy, 30 women with fetus suspected of having viral infection and 30 age-matched femal blood donors, had not yet been pregnant. Strain-specific antibodies against strains Towne (gB1) and AD169 (gB2) of HCMV were detected by ELISAs and microneutralization assay. The avidity of IgG was measured by a HCMV IgG avidity test.

The prevalence of Towne- and AD169-specific IgG of pregnant women proved to be 82.5% and 78.5%, respectively. Towne-specific IgG was found in 72% of blood donors. No detectable IgM antibodies were found in any of the pregnant women. The results showed prevalence of neutralizing antibodies in pregnant women 80% and 83.5% to Towne and AD169, respectively. Neutralizing antibodies were detected in 73% of blood donors against both strains of HCMV. No significant differences were found between the neutralizing antibody titers to Towne and AD169 in blood donors. The neutralizing capacity against both strains of HCMV was more effective in pregnant women with normal pregnancy than in blood donors. However, the neutralizing capacity to Towne strain was significantly lower than to AD169 in pregnant women with fetus suspected of having viral infection. The difference ranged from 4-fold to 16-fold in that neutralization titer.

According to the results of the IgG-avidity test most of the congenital infections originated from recurrent maternal infection.

Our data demonstrate significant differences in the prevalence and level of HCMV strain-specific neutralizing antibodies in pregnant women and age-matched female blood donors, which might have important implication for understanding of the pathogenesis of congenital HCMV infection and for development of anti-HCMV immunotherapy and vaccine.

2. Introduction

Human cytomegalovirus (HCMV) is a member of the *Herpesviridae* family. All known strains of HCMV are genetically homologous, but none seem to be genetically identical unless they are obtained from epidemiologically related cases [1, 2]. The sources of HCMV include urine, oropharyngeal, cervical and vaginal secretions, semen, breast milk, tears, blood products, and allografts [3-7]. HCMV is not higly contagious: spreading of the infection appears to require close or intimate contact of either a nonsexual or a sexual nature with another person who is shedding the virus in the bodily secretions. It can also be transmitted vertically, from mother to fetus. Infection with HCMV can be classified as either primary or recurrent. Studies on the age-related prevalence of infection with HCMV suggest that there may be three periods with particularly high rates of acquisition of the virus: early childhood, adolescence and the childbearing years. The prevalence of HCMV infection in the normal population varies widely between 40% and 90%, depending on the race, the gender, the age and the socioeconomic status [8]. HCMV is more prevalent among people in low socioeconomic brackets living in crowded conditions and in people living in undeveloped countries, with 80% of 3-year-old children and most adults in such groups being infected with HCMV [9, 10]. In Hungary, the seroprevalence of HCMV at the age of 10 years is 72%, which increases to 96% by the age of 50 [11]. After primary infection, the virus becomes latent, residing in the host throughout life. It is reactivated periodically during episodes of mild immunosuppression caused by intercurrent infection, pregnancy or stress. Recurrent infections are fairly common [12]. Intermittent excretion of the virus can be anticipated in a significant proportion of seropositive adults. Reinfection by a new strain of HCMV has been documented in immunocompromised individuals, women attending a clinic for sexually transmitted diseases and healthy children attending a day-care center [13-16].

Human cytomegalovirus rarely causes symptoms in an immunocompetent host, and these can be nonspecific symptoms, such as malaise, fever, sweats, aching muscles, atypical lymphocytosis and mild hepatitis during the self-limiting primary infection [17]. However, it can give rise to serious disease in an immunodeficient person, such as those with AIDS or neonates, and especially premature babies [12]. Reactivation is asymptomatic, except in severely immunocompromised individuals.

Human cytomegalovirus is the leading cause of congenital infection in developed countries, occurring in 0.3-2% of all live births [18]. To date very little research has been carried out on the natural history of intrauterine HCMV infection. In most cases the mother's infection was inapparent. The prevalence displays a specific geographical pattern: it is 0.9% in Hungary [12, 19]. Primary HCMV infection in adolescent females (aged 14-20) is mainly acquired by oral/sexual contact with saliva, genital secretions and semen, particularly in those of lower socioeconomic groups who change sex partners frequently [20, 21]. Women aged ≥25 years from the middle and upper socioeconomic classes, though not exempt from sexual promiscuity, may acquire infection predominantly by close contact with asymptomatic infants and toddlers who excrete HCMV in their saliva and urine [22, 23]. HCMV-seronegative women who work with children in day-care have an occupational risk of acquiring HCMV [24]. Fomites may also play a role in the transmission because HCMV has been shown to remain infective for hours on plastic surfaces [25]. This underlines the increased risk of acquiring HCMV infection to seronegative women or women planning pregnancy who are working in a child day-care setting. HCMV may also be transmitted and produce a congenital infection if a pregnant women or her fetus receives a blood product transfusion from a HCMV-seropositive donor.

Recurrent infections are common in pregnant women, and especially in a highly immune population, as estimated by the rate of congenital infection [12]. The most frequent mechanism for recurrent infection during pregnancy seems to be the reactivation of latent virus. However, the possibility of reinfection by HCMV strains other than the original infecting strain, particularly in women with multiple sexual partners, has been demonstrated by restriction enzyme analysis [26]. Recurrent infection occurs most frequently in the late second and third trimesters in seropositive women, when a marked transient depression of HCMV-specific cellular immunity can be demonstrated.

It has been reported that more than 10-15% of newborns infected congenitally are symptomatic at birth, and that 10-15% of the asymptomatic infected infants will develop long-term neurological sequelae, such as mental retardation, deafness, and visual impairment. This makes HCMV the leading infectious cause of central nervous system damage in children [27, 28]. Transmission of HCMV from mother to fetus can occur throughout gestation, and infection during the first 16 weeks of pregnancy has been associated with a higher incidence

of fetal damage [29, 30]. HCMV can also be transmitted to the fetus when primary maternal infection occurs before conception, but data are not available on the consequences for the newborn under these circumstances. Congenital HCMV infection can be a result of either exogenous or endogenous maternal infection. While exogenous infection can be primary or nonprimary, as observed in both seronegative and seropositive women, endogenous infection is a consequence of the reactivation of latent virus. Primary infection in the mother has a much greater clinical impact on the fetus than recurrent infection or exogenous reinfection. Primary infection with HCMV during pregnancy occurs in 0.7-4.1% of pregnancies, with a mean reported transmission rate to the fetus of 40% [31-33, 18, 34]. In contrast, the transmission rate during recurrent infection is much lower (1-2.2%) [31, 35].

HCMV is thought to be is transmitted when infected leukocytes cross the placental barrier to reach the fetal circulation via the umbilical cord vessels [36, 37]. Other routes may also be accessible to viral transmission. Even if *in vitro* data do not substantiate this hypothesis [38], one possibility is that the virus may first infect placental tissues and later amniotic cells. Consequently, infected amniotic cells would be ingested by the fetus, after which the virus could replicate in the oropharynx and invade the fetal circulation to reach target organs. The tubular epithelium within the kidney appears to be a major site of viral replication. By either mechanism of infection, the fetus would excrete HCMV via the urine into the amniotic fluid. The amniotic fluid therefore seems a logical choice of body fluid for the prenatal diagnosis of HCMV transmission [39-45]. In live-born neonates, demonstration of the virus in the urine within the first 3 weeks of life is an indication of congenital infection. This can be done by tissue culture or molecular methods.

To date, there is insufficient evidence to predict the consequences of HCMV infection of pregnant women, and the question of whether an infected fetus is to be damaged therefore remains unanswered.

Virological factor

HCMV glycoprotein B is the major envelope glycoprotein of HCMV and it is encoded by UL55 gene. HCMV gB has been implicated in host cell entry, cell-to-cell viral transmission and fusion of infected cells in addition to being an important target for both

antibody- and cell-mediated immune responses [46-48]. HCMV gB is expressed as a precursor molecule that is glycosylated and then cleaved at codon 461 to form a disulfide-linked complex of gp55 and gp116 [49]. Chou and Dennison devised a method of HCMV genotyping based on the gB nucleotide sequence that encodes a variable region encompassing the protease cleavage site [50]. They found that there were *Hinf*I and *Rsa*I restriction sites between nucleotides 1344 and 1440. Amplification of this region using the polymerase chain reaction (PCR) followed by restriction analysis demonstrated the existence of four different genotypes of gB [50]. Since gB has been implicated in host cell penetration, it is possible that the four types differ with respect to tissue tropism and virulence.

In recent years, several studies have suggested that HCMV strains with different gB genotypes are associated with different clinical outcomes of HCMV infection, as in bone marrow transplant recipients and in patients with acquired immunodeficiency syndrome (AIDS) and HCMV retinitis [51, 52]. In contrast, no such correlation was found in renal transplant patients [53, 54]. It remains unclear whether certain gB genotypes are associated with an increased frequency of disease.

Immunological factors

The humoral immune response is an important defense mechanism against human CMV. Unlike preconceptional immunity against rubella or toxoplasmosis, preconceptional immunity to HCMV provides only partial protection from intrauterine transmission of the virus. [55, 34, 35]. The level of HCMV-specific immunity among women of childbearing age varies widely among different populations. Seropositive rates in young women in the United States and Western Europe range from less than 50 to 85% [56, 57]. In contrast, in the Ivory Cost, Japan and Chile, the rate of seropositivity by the end of the second decade of life is greater than 90% [58-60].

Those infants who are congenitally infected with HCMV are mainly delivered from mothers having the first contact with HCMV or having reactivation during pregnancy. Between 0.7-4% of seronegative women are infected during pregnancy and it is known that pregnancy itself is not a risk factor for the infection [61]. This recognition has led to several studies to examine the possible factors and complex mechanisms that can determine the

pathogenesis of the infection during pregnancy. To come closer to this problem pregnant women must be divided into two groups concerning the pregnancy serological status. Congenital infection of the infant is most common among those circumstances when the mother is seronegative for HCMV before pregnancy and acquire the infection near conception or during the first 16 weeks of pregnancy. In these cases there are no maternal antibodies to bind to the virus therefore the risk of transmisssion is higher. In those women having recurrent infection, in the presence of specific immunity the transmisssion rate is approximately 1%, even when the transmission occurs, it is linked to less virulent outcome [62]. These infants are usually asymptomatic at birth and develop normally, although 90% of them shed the virus in urine for several months to years after birth. Approximatelly 10% of the infants will have hearing loss as the only manifestation of the disease [63].

Fowler et. al. reported that the transmission rate is 1.2% and 12.9% in seropositive and seronegative women, respectively, indicating that the preconceptional maternal immunity is protective against congenital infection, decreasing the risk by 90% [35]. However it is unknown why some women, even with recurrent infection and high levels of antibodies transmit the virus to the fetus, while others without antibodies during primary infection do not [64]. The transmission rate during trimesters is approximately the same but the outcome is different [63]. Only those infants have severe neurological disorders acquiring the infection near conception or during the first trimester.

Determination of the background immunity against HCMV is a key point concerning the outcome of the pregnancy and the possible infection of the fetus. The detection of antibodies against HCMV is clearly the indicator of infection. If the titer of IgG persists and do not show elevation, the individual is said to be seropositive and experienced the infection sometime in the past [65]. If the titer of IgG shows at least four-fold elevation or IgM is detectable in previously IgG-positive individuals, it suggests reactivation. Primary infection may also be documented through the detection of HCMV-specific IgG and IgM, although approximately 10% of individuals with primary infection will have negative IgM titer. Approximately 10% of women with recurrent HCMV infection also have positive antibody titer. IgM antibody may persist for up to 18 months after primary infections [63].

A reliable serological procedure to identify primary infection is determination of IgG avidity. The aim of the test is to examine whether the virus-specific antibodies are matured or

not. In the early stage of infection the existing antibodies do not bind to viral antigens exactly, but later by the activation of immunological response the antigen-specific antibodies are selected. A low IgG avidity is a marker of primary HCMV infection for 18-20 weeks after onset of symptoms in immunocompetent host, a high avidity is related to infection in the past [62]. In Figure 1. there are proposed schemes for diagnosis of pregnant women, according to their serological status for cytomegalovirus (62).

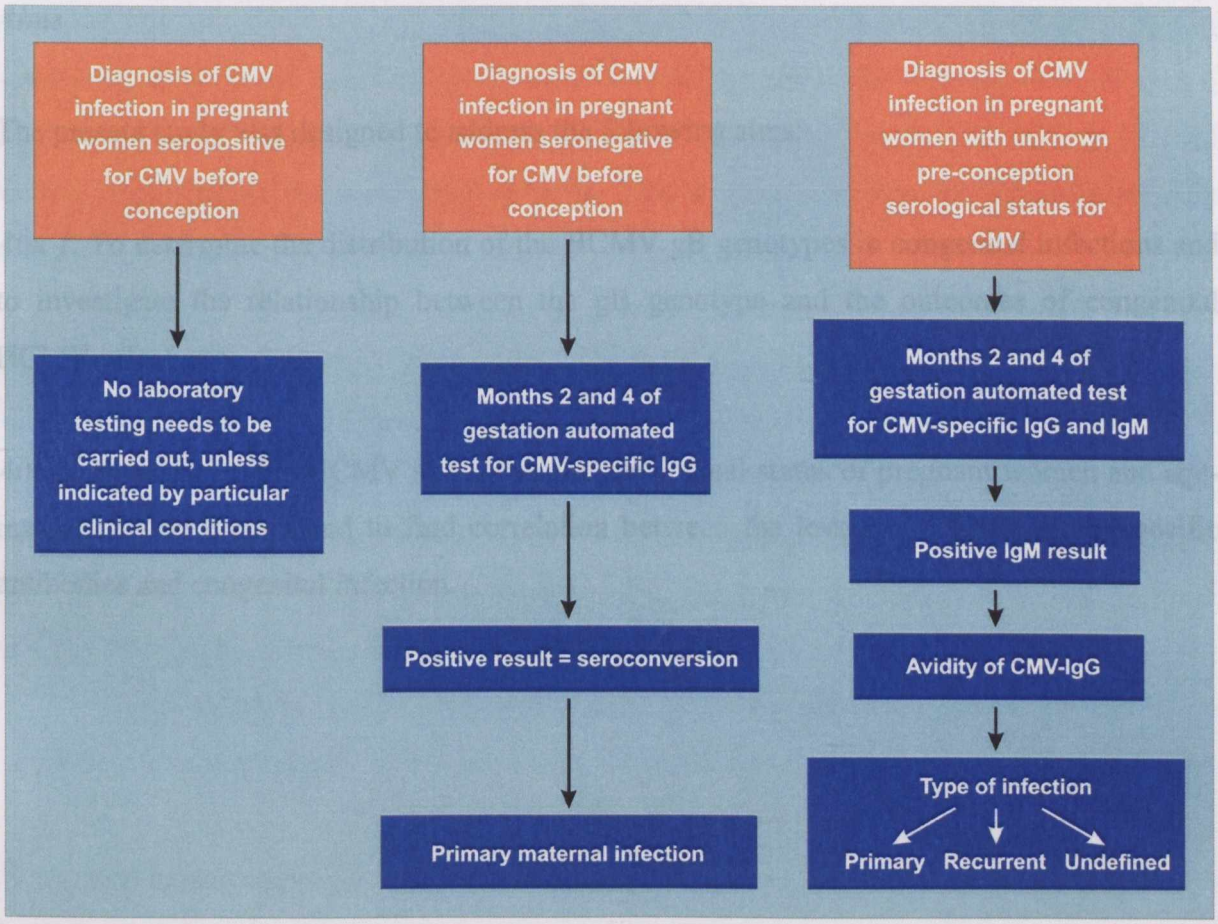


Figure 1:
Proposed schemes for diagnosis of pregnant women, according to their serological status for cytomegalovirus (CMV): seropositive for CMV before conception; seronegative for CMV before conception, and unknown pre-conception serological status.

Detection of HCMV-specific IgG antibodies does not mean that they have neutralizing activity. The major target of neutralizing antibodies is the envelope glycoprotein B by which the virus can be classified into four genotypes (gB 1-4), as described above [50]. Neutralizing antibodies first appear approximately 15 weeks after acute infection [8]. It means that those antibodies detected earlier by very sensitive methods such as ELISAs are produced against nonglycosilated proteins which appear earlier and they have no neutralizing activity. This fact could explain the discrepancy between the different results of ELISAs and neutralization assays carried out in the early stage of acute infection.

Aims

The present study was designed to address the following aims:

Aim 1. To determine the distribution of the HCMV gB genotypes in congenital infections and to investigate the relationship between the gB genotype and the outcomes of congenital HCMV infections.

Aim 2. To examine the HCMV strain-specific serological status of pregnant women and age-matched blood donors and to find correlation between the levels of HCMV strain-specific antibodies and congenital infection.

3. Materials and Methods

Patients and samples

Sixty-three pregnant women who were referred to the Department of Gynecology and Obstetrics of the University of Szeged between January 1996 and August 1998 were included in the study. In 40 pregnancies (gestational age: 27±8.4 weeks), the fetuses or newborn babies were strongly suspected of having viral infections (suspected group). The suspicion of virus infection arose in consequence of the detection of an abnormality by the ultrasound scan, such as intrauterine growth retardation (IUGR), nonimmune hydrops fetalis, ascites, hydrothorax, hydrocephaly, microcephaly, spina bifida, hepatomegaly, hyperechogenic bowel, or a heart or kidney anomaly. Those cases of spontaneous abortion or premature delivery that involved a suspicion of viral infection were also included. Twenty-three women at no risk of fetal HCMV transmission, but from whom amniotic fluid was taken for fetal karyotype assessment constituted the control group (gestational age: 17.3±1.8 weeks).

A total of 60 amniotic fluid samples were examined; 37 of these were from the suspected group, and 23 from the healthy pregnant women. From 8 pregnancies, fetal (n=4) and newborn (n=6) blood samples were obtained. A total of 12 urine samples from 6 newborns were also included in the study. The urine samples of infants suspected of having viral infection were examined for HCMV DNA within the first 2 weeks of life. We also detected HCMV DNA from blood samples of the mothers where available, and from placenta samples from 6 pregnancies from the suspected group.

Ethical approval for this study was granted by the Human Investigation Review Board of the University of Szeged.

Processing of specimens

Amniotic fluid and urine samples were aliquoted into Eppendorf tubes or centrifuged at 300 g for 5 min, and the supernatant and sediment were stored at –70°C until they were processed. Peripheral blood was collected in anticoagulant-containing vacuetta tubes (Greiner, Germany). Peripheral blood leukocytes were isolated by centrifugation of whole

blood at 300 g for 5 min, with subsequent lysis of the erythrocytes. The peripheral blood leukocytes were pelleted, washed 3 times with phosphate-buffered saline (PBS), pH 7.4, and quantitated. Peripheral blood leukocytes suspensions were adjusted with PBS to a concentration of 10^6 peripheral blood leukocytes per ml. Aliquots of $5x10^5$ peripheral blood leukocytes were centrifuged at 550 g for 5 min. The supernatant was discarded and the cells were stored at –70°C until use. Placenta samples were stored at –70°C until they were processed.

DNA preparation

Aliquots of $5x10^5$ peripheral blood leukocytes were resuspended in 200 µl of PBS. HCMV DNA was individually extracted from at least 3 aliquots of amniotic fluid, urine, peripheral blood leukocytes and placentas (200 µl each), using the High Pure Viral Nucleic Acid Kit (Boehringer Mannheim GmbH, Indianapolis, IN, USA) according to the manufacturer's instructions, and was resuspended in 50 µl of nuclease-free distilled H_2O.

Polymerase chain reaction amplification of gB gene and restriction analysis

A region of high peptide variability in the gB gene was amplified. To improve the sensitivity and to overcome sequence variation between strains, a nested PCR was used. Primers for amplification were selected from the published gB sequences of HCMV strain AD169 [50]. The primers were as follows: outer primers: gB 1246, 5'-GGAAACGTGT CCGTCTTTGA; gB 1724, 5'-GAGTAGCAGCGTCCTGGCGA; internal primers: gB 1319, 5'-GGAACTGGAACGTTTGGC; gB 1604, 5'-GAAACGCGCGGCAATCGG. The first round of amplification was carried out in a total volume of 50 µl, using 5 µl of DNA extract, PCR mix I, consisting of 10x Reaction Buffer (Pharmacia Biotech, Uppsala, Sweden), sterile H_2O, supplemented with 0.4 µM of each oligonucleotide primer; and PCR mix II, consisting of 200 µM of each of the four dNTPs, 1.25 U of Taq polymerase (Pharmacia Biotech, Uppsala, Sweden), 10x Reaction Buffer, distilled H_2O. The second round of amplification was carried out in a total volume of 50 µl, 3 µl of the first-round amplification product being added to 25 µl of PCR mix I. 20 µl of PCR mix II was added to each sample. The samples

were covered with mineral oil (Sigma, Deisenhofen, Germany), denatured (95°C, 300 sec), and amplified in 35 cycles (95°C, 30 sec; 55°C, 40 sec; 72°C, 60 sec). Amplification products (293-296 bp, the size varying with the strain) were analyzed by electrophoresis in a 2% agarose gel (BRL, Gaithersburg, Germany) stained with 5 μl of 10 mg/ml ethidium bromide (Serva Feinbiochemica, Heidelberg, Germany) and subjected to restriction analysis by using *Hinf*I and *Rsa*I (Promega, Madison, WI, USA) as previously described [50]. Digested DNA was analyzed in a 3% agarose gel (Nu Sieve 3:1, FMC Bio Products, Rockland, ME, USA). Four distinct gB genotypes can be identified via the different lengths of restriction fragments (36-239 bp) [50].

DNA from the Towne HCMV strain (gB genotype 1) was used as a positive control, and a sample without any DNA as a negative control.

Immunological methods

Study population and clinical specimen

Pregnant women (n=60) who were referred to the Department of Gynecology and Obstetrics of the University of Szeged between January 1996 and August 1998 were included in the study; 30 pregnant women (mean age: 28.7±6.6 years) with normal pregnancies (gestational age: 29.7±11.3 weeks), and 30 women (mean age: 27.4±4.6 years) with fetuses suspected of having viral infection (gestational age: 27.8±8.7 weeks). The study population consisted also of 30 age-matched blood donors, as controls (mean age: 26.9±7 years) with no history of pregnancy (Table 1. and 2.). Plasma or serum samples of women were collected and stored at -20°C until examination.

Cell culture and virus

Human embryo fibroblast (MRC-5) cells were obtained from the Wistar Institute, Philadelphia, PA, USA. Cells were propagated in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (Gibco Life Technologies Inc., Paisley, Scotland), penicillin (100 U/ml),

streptomycin (100 μg/ml) in 75 cm^2 tissue culture flasks (Corning Costar Corporation, Oneonta, NY, USA) at 37°C in a humidified chamber with 5% CO_2 and passaged weekly.

Towne (gB1) and AD169 (gB2) laboratory strains of HCMV were obtained from the Wistar Institute, Philadelphia, PA, USA, and propagated in MRC-5 cells by standard procedure and stored in 1 ml aliquots in liquid nitrogen [66]. The infectivity of virus was determined by plaque assay [67].

Table 1. Age distribution of women with normal pregnancy and with fetuses suspected of having viral infection and blood donors.

Women	**Age (year)**			
	18-24	**25-29**	**≥30**	**Mean ± SD***
With normal pregnancy	9	8	13	28.7 ± 6.6
With fetus suspected of having viral infection	10	11	9	27.4 ± 4.6
Blood donors	13	9	8	26.9 ± 7.0

*standard deviation

Table 2. Gestational age of pregnancies at collection of specimens.

Pregnancies	**Gestational age (week)**			
	≤14	**15-28**	**≥29**	**Mean ± SD***
Normal	0	13	17	29.7 ± 11.3
Fetus suspected of having viral infection	3	10	17	27.8 ± 8.7

*standard deviation

Enzyme-linked immunosorbent assay

All samples were screened by two different types of ELISAs.

Type 1:

ELISA (in house) for detecting antibodies to Towne strain of HCMV were performed as described by Gönczöl et al [68]. A preparation of semipurified Towne strain of HCMV was used as coating antigen in assays to detect Towne strain-specific serum antibody. After coating the wells, plates were blocked with PBS containing Tween 20 (0.05%, v/v) and skim milk (1% w/v) for one hour. After discarding the blocking solution the plates were washed three times with PBS containing Tween 20. For antibody testing, plasma or serum samples and horseradish peroxidase HRP-labeled anti-human IgG (Sigma) were diluted in blocking buffer containing skim milk (0.4%) and incubated sequentially on the plates. After washing, bound peroxidase was detected by adding substrate (0.1 M citrate buffer (pH 4.5) containing hydrogen peroxide (0.012%, v/v) and 0-phenylene-diamine 1 mg/ml) for 10 min, at which point the reaction was stopped with 4 M sulfuric acid. The optical density (OD) at 490 nm was read with a microplate reader (Stat Fax-2100, Awaraness Technology Inc. Palm City, FL, USA). The cutoff value for antibody positivity was defined as the mean OD of five seronegative sera plus 3SD.

Type 2:

Commercially available ELISA kits (Enzygnost Anti-CMV/IgG and Enzygnost Anti-CMV/IgM kits, Dade Behring Marburg GmbH, Marburg, Germany) were used. The ELISAs were manually performed traditional indirect assays in which microtiter plate wells were coated with purified HCMV (strain AD169) and the results were interpreted according to the manufacturer's recommendations. Serum samples were pretreated by rheumatoid factor adsorbent prior to testing with ELISA for CMV-IgM. Serum samples were diluted in the respective test buffers and incubated in a humidified chamber for 60 min at 37°C. The plate was washed with washing buffer 3 times. Then 100 µl of horseradish peroxidase conjugated goat anti-human IgM or IgG was added. After 60 min incubation at 37°C the plate was washed 3 times and 100 µl of Chromogen TMB was added into all wells and incubated for 30 min at room temperature. 100 µl of stopping solution blocked the

reaction. The evaluation was carried out within 60 min. The assay wavelength to be used was 450 nm.

Avidity of IgG

The determination of IgG antibody avidity was carried out by the urea determination procedure using a kit recently made available (Cytomegalovirus IgG Avidity EIA WELL, produced by RADIM, Rome, Italy). The coating-antigen is characterized by an HCMV strain AD169. The test is based on the difference between the absorbance values due to antibody binding in the absence and in the presence of 4.5 M urea. The test was run and results were interpreted as it is suggested by the manufacturer. Briefly, 10 µl of sera were diluted in 3 ml sample diluent. Control samples of high and low avidity indices were included in each test run. 100 µl of dilution of serum to be tested were added to the antigen-coated wells. After an hour incubation in a humidified chamber at 37°C the plate was washed four times with 350 µl of washing solution (PBS Tween:distilled water = 1:20). After discarding the washing solution 100 µl sample diluent (into the odd wells) and 100 µl of dissociating reagent were added into the even wells and incubated for 30 min at 37°C. The plate was washed 4 times and 100 µl of enzyme tracer was added into all wells and incubated for 30 min at 37°C. After washing four times 100 µl of freshly prepared substrate solution was added into all wells and incubated for 10 min at 37°C. After incubation 100 µl of blocking reagent was pipetted into all wells. The OD at 450 nm was read with a bichromatic spectrophotometer within 60 minutes. CMV-specific IgG avidity index (AI) expressed as follows: percentage of AI = (absorbance results of CMV for well with urea wash/absorbance results of CMV for well without urea wash) x 100. An AI lower than 35% was considered low and indicates acute primary infection, index of 35-45% is considered borderline and an avidity index higher than 45% was considered high indicating past infection.

Microneutralization assay

Neutralizing antibodies to Towne and AD169 strains of HCMV were measured by a rapid microneutralization assay. HCMV neutralization test was carried out as described by Gönczöl et. al. [69]. Briefly, 96-well, sterile, flat bottomed culture plates were filled with 60 µl RPMI 1640 supplemented with 5% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 µg/ml) except the first line wells. The first line was filled with 90 µl RPMI 1640 and equal volume of plasma or serum samples (30 µl) were added to each and serial two-fold dilutions were made. Control wells received culture medium only. Then the virus and complement suspension was added. Each well contained 5000 plaque forming unit (PFU) of the virus. After an hour long incubation at 37°C, 60 µl cell suspension ($3\text{-}5x10^4$ cell/well) was added to each well and incubated for additional 48 hours. The results were determined with the help of positive and negative controls in which the cells and the cytopathic effect (CPE) of the virus can be evaluated. The reciprocals of the highest dilution of plasma or serum that inhibited 90% of the viral CPE were reported as the neutralizing titer.

Statistical analysis

Owing to the non-normal distribution of neutralizing antibody titers nonparametric test were suitable. For the global analysis of data the Kruskal-Wallis nonparametric ANOVA test was used. Pairwise comparisons were tested using the Mann-Whitney U-test. When multiple comparisons were performed, we applied Bonferroni correction. Two-tailed p values are given throughout the test. Two-tailed p values of <0.05 were considered to be significant. All the calculations were performed on a SPSS 9.0 software.

4. Results

Aim 1. *To determine the distribution of the HCMV gB genotypes in congenital infections and to investigate the relationship between the gB genotype and the outcomes of congenital HCMV infections.*

Forty pregnancies in which the fetuses were suspected of having a viral infection and 23 normal pregnancies were examined for congenital HCMV infection. The presence of HCMV DNA was detected by means of a nested PCR specific for the nucleotide region between bases 1319 and 1604 of the gB gene in the samples of amniotic fluid, fetal or newborn blood, blood of the mother, urine of the fetus or newborn, and placenta. HCMV was found in 12 (30%) of the pregnancies in the suspected group, and in the amniotic fluid samples from 3 women with normal pregnancies (13%) (Table 3.).

Table 3. Congenital HCMV Infection Detected by PCR.

Pregnancies	**Gestational age (weeks)**	**HCMV DNA**	
		Positive	**Negative**
Fetus suspected of having viral infection*	27±8.4	12	28
Normal	17.3±1.8	3	20

*Intrauterine growth retardation, nonimmune hydrops fetalis, hydrocephaly, microcephaly, spina bifida, hyperechogenic bowel, heart or kidney anomaly

Genotyping of HCMV DNA from the amniotic fluid, urine and peripheral blood leukocyte samples was undertaken by restriction fragment length polymorphism (RFLP). All the HCMV DNA had identical genotype, gB1. The gB restriction endonuclease digest patterns of amplimers from the Towne strain (gB genotype 1) and that from one of the amniotic fluid samples in the suspected group are shown in Figure 2.

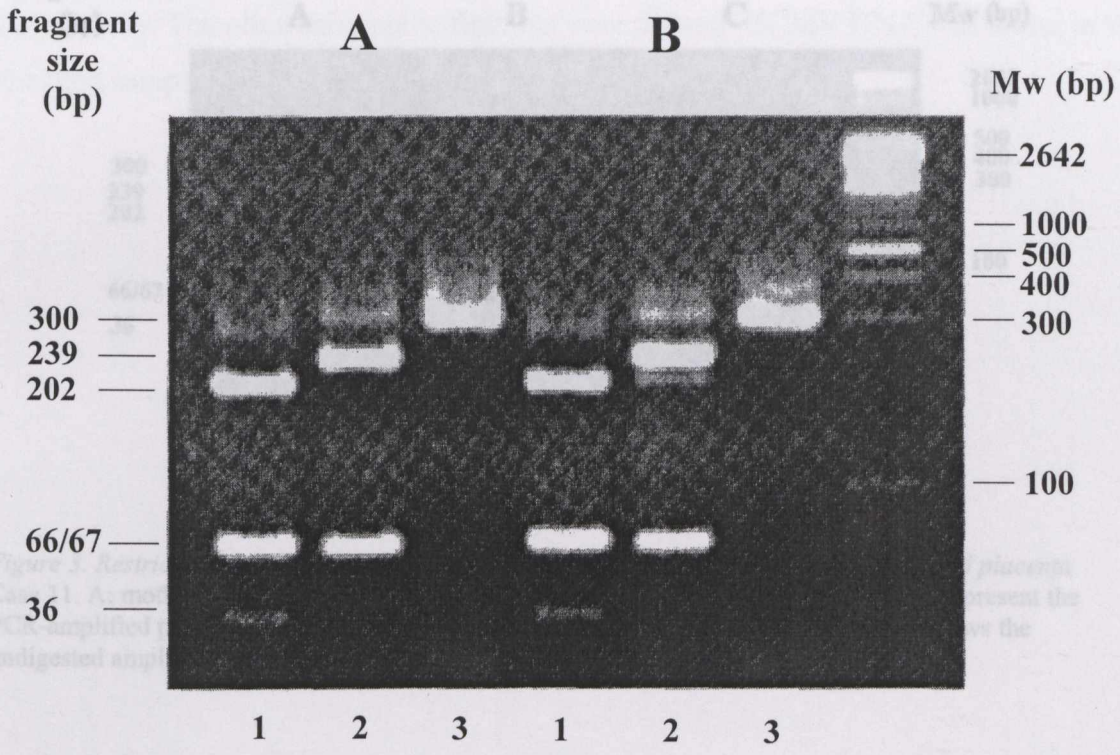


Figure 2. Restriction patterns of gB PCR products from two HCMV DNA.
A: DNA from Towne strain. B: HCMV DNA from case 11. Lanes 1, 2, PCR-amplified part of gB cut with *Hinf* I and *Rsa*I, respectively; lane 3, undigested amplimers. Mw, molecular weight markers.

In the matched samples from the mother-infant pairs, the HCMV genotypes were similar. The restriction enzyme patterns of the gB PCR products from the mother's blood, the amniotic fluid and the placenta in case 11 are shown in Figure 3. None of the mothers were infected with more than one gB genotype.

Information concerning the infected fetal and neonatal outcomes was obtained directly when the mothers had terminated the pregnancies or given birth. The infection status of the neonates was classified on the basis of the detection of viral DNA within 2 weeks after birth. The infected neonates were further classified as having symptomatic or asymptomatic infection.

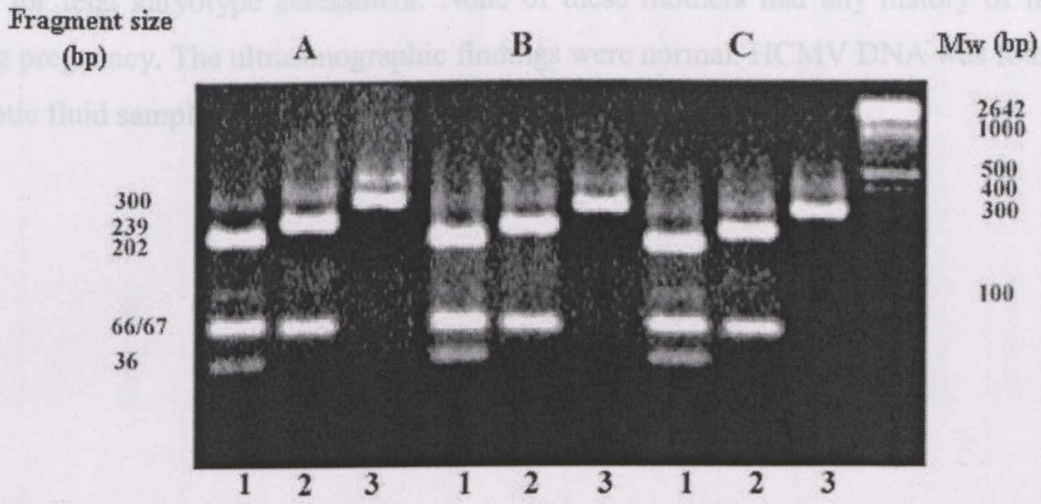


Figure 3. Restriction patterns of gB PCR products from mother's blood, amniotic fluid and placenta
Case 11. A: mother's blood, B: amniotic fluid, C: placenta; Lanes 1 and 2 of each panel represent the PCR-amplified part of gB cut with *Hinf* I and *Rsa*I, respectively; Lane 3 of each panel shows the undigested amplimers. Mw, molecular weight markers.

The clinical outcomes of the pregnancies of the 12 women in the suspected group whose fetuses or newborns proved to be infected *in utero* are shown in Table 4. Congenital HCMV infection was observed in 10 fetuses and in 6 neonates (4 of the pregnancies had twins). The ultrasonographic findings were abnormal in 9 pregnancies involving 11 fetuses. Six of the 9 cases (cases 1, 2, 6, 8, 9, and 10) were detected between 31 and 38 weeks gestation. The newborns (n=7) were HCMV-infected, 3 without symptoms and 4 with symptoms. Of the 3 remaining cases, which were between 19 and 24 weeks gestation, case 5 was a twin bichorionic biamniotic pregnancy. At 21 weeks gestation, the ultrasonographic scan revealed that one of the twins was anencephalic, and elective abortion was carried out. In case 11, the fetus was hydrocephalic; this was detected at 24 weeks of gestation and the decision was made to terminate. Necropsy revealed internal hydrocephaly, corpus callosum agenesia, cerebellum malformations, and focal lesions in the placenta. In case 12, with polycystic kidney, the pregnancy was likewise terminated. In 3 cases (cases 3, 4, and 7), ultrasound examinations did not detect any anomalies. The outcomes of these pregnancies were spontaneous abortion (case 3) or premature delivery (cases 4 and 7).

The control group consisted of 23 women from whom samples of amniotic fluid were taken for fetal karyotype assessment. None of these mothers had any history of infections during pregnancy. The ultrasonographic findings were normal. HCMV DNA was found in the amniotic fluid samples from 3 of the 23 pregnancies (Table 3).

Table 4. Characteristics and Outcomes of Pregnancies with gB Genotype 1 HCMV Infection.

Case number	Weeks of gestation	Ultrasonographic findings	HCMV DNA			Outcome
			Amniotic fluid	Blood of fetus (F) or newborn (N)	Urine of infant	
1	31	Nonimmune hydrops fetalis	+	+ (F)	+	Symptom-free CMV-infected neonate, week 36.*
2	38	Intrauterine growth retardation	+	+ (N)	ND	Small for gestational age, CMV-infected neonate.
3	21	Twins	+	ND	ND	Spontaneous abortion.
4	24	Twins	"A": ND "B": ND	+ (N) + (N)	ND +	Premature delivery at week 24.*
5	21	Twins "A": normal "B": anencephaly	ND +	- (N) + (F)	+ ND	Symptom-free, CMV-infected neonate, week 36.* Selective abortion at week 21.
6	36	Discordant twins	"A": ND "B": ND	ND ND	+ +	Small for gestational age, CMV-infected neonate. Symptom-free CMV-infected neonate.
7	32	Normal	+	ND	ND	Premature delivery.
8	35	Hepatomegaly	+	+ (N)	+	Symptom-free CMV-infected neonate, week 39.*
9	31	Spina bifida, hyperechogenic bowel, microcephaly	+	+ (N)	ND	CMV-infected neonate with spina bifida and microcephaly, week 41.*
10	34	Nonimmune hydrops fetalis	+	+ (F)	ND	Nonimmune hydrops fetalis, week 34.*
11	24	Hydrocephaly	+	+ (F)	ND	Termination of pregnancy.
12	19	Polycystic kidney	+	ND	ND	Termination of pregnancy.

ND, not done; * time of delivery.

Aim 2. To examine the HCMV strain-specific serological status of pregnant women and age-matched blood donors and to find a correlation between the levels of HCMV strain-specific antibodies and congenital infection.

Cytomegalovirus strain-specific humoral immunity was investigated in pregnant women and age-matched blood donors by using two laboratory strains of HCMV: Towne (gB1) and AD169 (gB2). The prevalence of strain-specific IgG and of neutralizing antibody activity, the level of neutralizing antibodies and the IgG avidity were measured in serum and plasma samples. The study group consisted of 30 women with normal pregnancies, 30 women with fetuses suspected of having a viral infection and 30 age-matched blood donors who had not yet been pregnant (Tables 1. and 2.).

Prevalence of IgG antibodies against strains Towne and AD169

The prevalence of IgG antibodies against different strains of HCMV was tested in two types of ELISA. Strain-specific antibodies against strain Towne were measured in all groups of women involved in this study. A detectable amount of antibodies against strain Towne was found in 79% of the women. However, IgG antibodies against Towne were exhibited by a higher percentilage of the pregnant women (82.5%) than of the blood donors (72%). No difference was observed in the prevalence of Towne-specific IgG between the groups of pregnant women (Table 5).

The prevalence of antibodies against strain AD169 was investigated only in the pregnant women. Antibodies against this strain were detected in 78.5% of them (Table 5). No significant difference was found in the prevalence of these strain-specific antibodies between the groups of pregnant women. None of the pregnant women had HCMV-specific IgM.

Table 5. Prevalence of HCMV-specific IgG and IgM tested by different ELISAs.

Women	ELISA (in-house)[1]	ELISA (Behring)[2]	
	IgG	IgG	IgM
	No. positive/No. tested (%)		
With normal pregnancy	23/28 (82)	24/30 (80)	0/30 (0)
With fetus suspected of having viral infection	25/30 (83)	23/30 (77)	0/30 (0)
Blood donors	21/29 (72)	NT[3]	NT[3]

[1]Towne-specific; [2]AD169-specific; [3]not tested.

Prevalence of HCMV strain-specific neutralization antibodies

Neutralizing antibodies against strain Towne were detected in 78% of all the women investigated (n=90), and 80% of them displayed neutralizing antibody activity against strain AD169. 80% of the pregnant women and 73% of the blood donors exhibited neutralizing antibodies against strain Towne (Table 6). The prevalence of neutralizing antibodies against AD169 was 83.5% and 73% in the pregnant women and in the blood donors, respectively. Thus, the levels of prevalence of neutralizing antibodies against the two different strains of HCMV in the pregnant women and in the blood donors were very similar. Furthermore, there was no difference in the prevalence of neutralizing antibodies against strain Towne between the women with normal pregnancies and the women with a fetus suspected of having viral infection (80% in each group). There was a moderate difference in the prevalence of neutralizing antibodies against AD169 between the normal and the suspected pregnant group (80% and 87%, respectively) (Table 6).

The prevalence of neutralizing antibodies against strain Towne obviously

increased with age (68%, 74% and 95%) in the three different age groups of the pregnant women (Figure 4). As even higher tendency was observed as concerns the prevalence of neutralizing antibodies against strain AD169 (74%, 79% and 95%). Such a regularity was not detected in the blood donors. The levels of prevalence of neutralizing antibodies were similar within the age groups 18-24 and 25-29 years, but there was an obvious difference between the pregnant women and the blood donors in the third age group (>29 years), where the prevalence of Towne-specific neutralizing antibodies was 95% in the pregnant women and 75% in the blood donors.

Table 6. Prevalence of neutralizing antibodies against strains Towne and AD169 of HCMV.

Women*	Neutralizing antibodies against	
	Towne	AD169
	No. positive (%)	
With normal pregnancy	24 (80)	24 (80)
With fetuses suspected of having viral infection	24 (80)	26 (87)
Blood donors	22 (73)	22 (73)

*30 women in each group

Strain-specific neutralizing activity

To analyze the levels of neutralizing antibodies to strains of HCMV in the groups of pregnant women, two tests were used. With the Kruskal-Wallis test, the difference between the means of the neutralizing antibody titers in the groups was very significant: $p=0.0075$.

The Mann-Whitney test too was used to compare the neutralizing antibody levels in the two groups. Figure 5 shows the neutralizing activity against strains Towne

(gB1) and AD169 (gB2) in the blood donors (group 1), the women with normal pregnancies (group 2), and the women with fetuses suspected of having a viral infection (group 3).

Figure 4. Wariation with age of neutralizing antibodies against strains Towne and AD169 in pregnant women and blood donors.

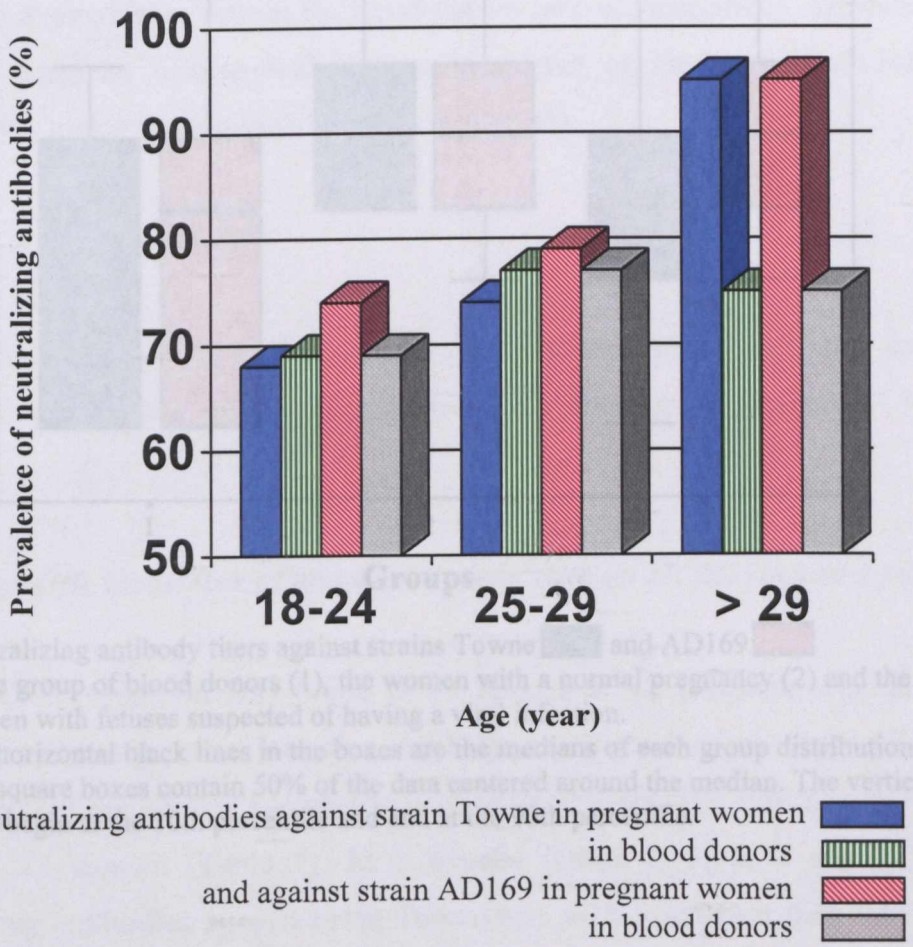
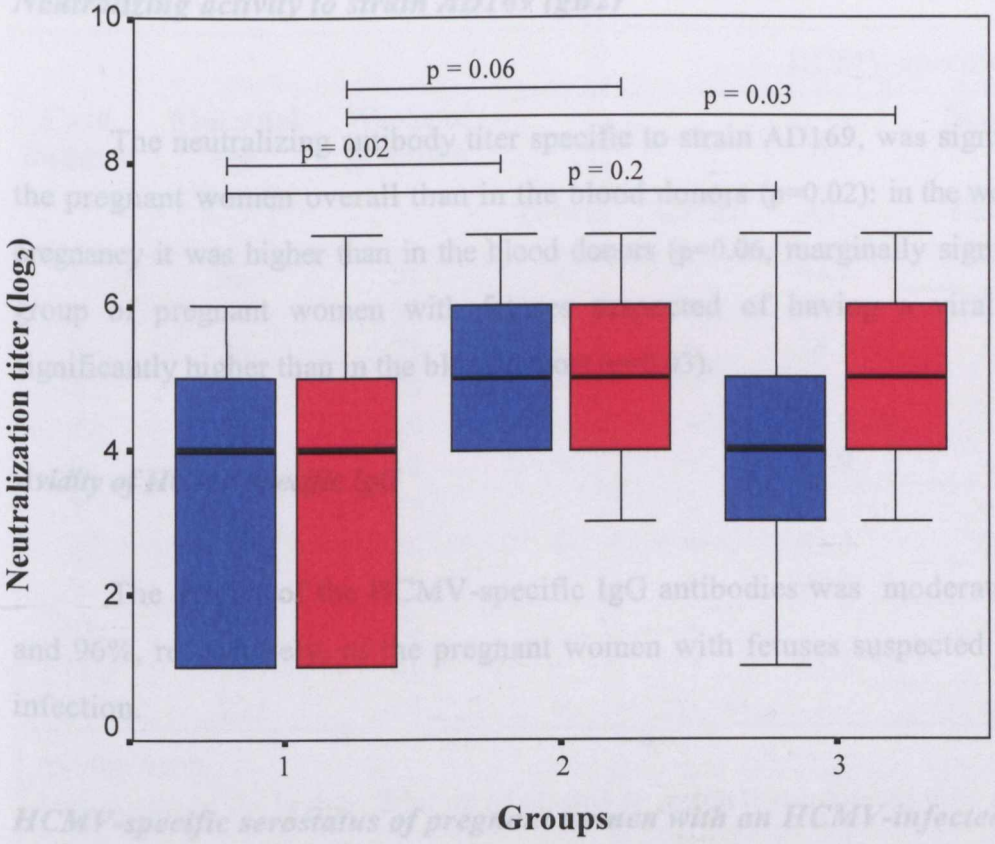


Figure 5. Neutralizing activity against strains Towne (gB1) and AD169 (gB2) in pregnant women and blood donors.



Neutralizing antibody titers against strains Towne and AD169 in the group of blood donors (1), the women with a normal pregnancy (2) and the women with fetuses suspected of having a viral infection.
The horizontal black lines in the boxes are the medians of each group distribution. The square boxes contain 50% of the data centered around the median. The vertical lines begin at the 10th percentile and end at the 90th percentile.

Neutralizing activity to strain Towne (gB1)

The neutralizing titers were significantly higher in the pregnant women (group 2 and 3) than in the blood donors (group 1) (p=0.04). However, there was a significant difference in neutralizing antibody titers only between the blood donors and the women with normal pregnancies (p=0.02), i.e. the level of neutralizing antibodies to strain Towne was lower in the blood donors. The mean of the neutralizing antibody titers in the women with fetuses suspected of having a viral infection was similar to that in the blood donors (p=0.2).

Neutralizing activity to strain AD169 (gB2)

The neutralizing antibody titer specific to strain AD169, was significantly higher in the pregnant women overall than in the blood donors (p=0.02): in the women with a normal pregnancy it was higher than in the blood donors (p=0.06, marginally significant), but in the group of pregnant women with fetuses suspected of having a viral infection it was significantly higher than in the blood donors (p=0.03).

Avidity of HCMV-specific IgG

The avidity of the HCMV-specific IgG antibodies was moderate and high in 4% and 96%, respectively, of the pregnant women with fetuses suspected of having a viral infection.

HCMV-specific serostatus of pregnant women with an HCMV-infected fetus

HCMV infection was found in 16 fetuses of 12 pregnant women with fetuses suspected of having a viral infection. All these cases involved genotype gB1. At the time of detection of the clinical symptoms, neither HCMV-specific IgM nor IgG was found in 3 of the 12 women (Table 7). In 5 women (cases 1, 5, 6, 9 and 11) the titer of neutralizing antibodies against strain Towne was 4-16-fold lower than that against strain AD169 (compared on an individual basis). In the sera of most of the women with a normal pregnancy, the titers of neutralizing antibodies against strains Towne and AD169 did not differ significantly (equal to or less than 4-fold).

A significant difference in the neutralizing titers against strains Towne and AD169 in the sera of 6 blood donors was observed. These ranged from 4-fold to 8-fold. In 5 cases a higher neutralization capacity was found against strain AD169, while in 1 case the Towne-specific neutralization capacity was significantly higher.

Table 7. HCMV-specific serostatus of pregnant women with an HCMV (gB1)-infected fetus.

Case number	Maternal age (year)	Weeks of gestation	HCMV-specific				
			IgG		IgG	Neutralizing antibody titer[2]	
			Towne	AD169	AI[1]	Towne	AD169
1	42	31	+	+	62.7	16	64
2	25	38	+	+	58.8	64	128
3	24	21	+	+	38.8	16	32
4	30	25	+	+	68.4	32	32
5	26	19	+	+	60.6	32	128
6	25	12	+	+	59.9	<4	16
7	24	21	-	-	-	<4	<4
8	33	36	+	+	62.2	64	64
9	25	32	+	+	90.2	64	512
10	27	35	-	-	-	<4	<4
11	24	41	+	+	63.3	16	128
12	29	13	-	-	-	<4	<4

[1] Avidity index.
[2] Reciprocals of the highest dilution of plasma or serum that inhibited 90% of the viral CPE.

5. Discussion

The factors that influence the transmission of HCMV to the fetus and mediate fetal damage have not been fully established. A preexisting HCMV-specific humoral immune response can lessen the deleterious effects on the fetus. However, in contrast with congenital rubella or toxoplasmosis, the maternal antibody does not completely protect the fetus [70]. Thus, although congenital HCMV infection is more likely in a seronegative pregnant woman who has a primary HCMV infection during pregnancy, HCMV-infected infants can also be born to women with recurrent disease [71-73]. Symptomatic infection occurs in 5-10% of infants infected congenitally, with more than 90% of these children experiencing motor, visual, cognitive, or auditory sequelae. The remaining 90% of infants infected congenitally will have no obvious signs or symptoms of HCMV, but many will develop a sensorineural hearing loss subsequently [27, 74, 75].

Prenatal diagnosis of congenital HCMV infection can be carried out by culturing or the PCR to detect the HCMV in the amniotic fluid and to distinguish uninfected from infected fetuses. However, the outcome of the infection cannot be predicted [39-45]. HCMV is a virus that grows relatively slowly in vitro, and Apperley and Golman has shown that the virus could not be detected in the blood circulation until 2 or 3 weeks after the primary infection [76]. Four to six more weeks are necessary for the virus to contaminate the fetus, proliferate in its tissues and finally occur in urinary excretion in amniotic fluid. It, therefore, follows that in most cases at least 6 weeks elapse are necessary after the mother's primary infection before HCMV can be in the amniotic fluid. Moreover, it seems that the stage of pregnancy at the date of amniocentesis influences the sensitivity of the antenatal diagnosis [77, 78]: when the mother's seroconversion occurs during the first 3-month period, a longer delay may be necessary before the viral growth becomes detectable in the fetal compartment.

distribution of the gB genotypes in congenital infections indicate that HCMV strains of diverse genotypes can be transmitted vertically, and strains bearing the gB1 genotype are predominant in different geographical regions of the world [84].

The present group of patients were mainly from the city of Szeged; some of the severe cases were from three neighboring counties in south Hungary. The gB RFLP of the HCMV DNA from the samples revealed that they were homologous. The dominance of gB genotype 1 could reflect the infection type of the study population. No data on the overall distribution of HCMV gB genotypes for populations other than fetuses and newborns are available in Hungary.

In this study, HCMV DNA was found not only in the samples from pregnancies in the suspected group, but in 3 of 23 normal pregnancies too. Kyriazopoulou et al. published a similar observation: HCMV DNA was detected in the amniotic fluid from 4 of 32 pregnant women with normal ultrasonographic findings. These women were HCMV seropositive before conception. The preconceptional HCMV serostatus of the women in the current study was not known, but at the time of amniocentesis they had HCMV-specific neutralizing antibodies. These results draw attention to the fact that fetal HCMV infection is not rare among pre-immune women [85].

A previous study demonstrated a correlation between the gB genotype and the severity of HCMV disease in bone marrow transplant recipients [51]. The patients who survived HCMV infections after bone marrow transplantation were more likely to excrete strains with the gB1 genotype than were patients who died. Shepp et al. reported that the presence of gB2 in blood isolates was potentially associated with a greater risk of the development of HCMV retinitis in patients with AIDS [52]. Another study found no association between HCMV gB genotypes and CMV-related central nervous system disease. The HCMV gB types 1 and 2 were the HCMV genotypes most frequently found, both in the CSF of AIDS patients with CMV-related neurological disease (42.3% and 26.9%, respectively) and in the urine and saliva of AIDS patients without CNS disease (42.8% and 23.8%, respectively) [86]. It has been reported that there is no correlation between HCMV gB genotypes and CMV disease in renal transplant patients [53, 54]. However, another study suggests that HCMV gB genotype 2 is present most frequently in the same group of patients [87]. The gB genotypes did not correlate with the neurodevelopmental outcome of intrauterine infection [83]. In Toronto,

Canada, Sarcinella et al. found that the distribution of HCMV gB genotypes in liver transplant patients was: gB1 (25.9%), gB2 (27.6%), gB3 (36.2%), gB4 (3.4%) and four patients (6.9%) had mixed infection. Symptomatic CMV disease developed in 43.1% of patients and 44.8% of them had acute rejection. The rate of CMV disease and acute graft rejection in patients infected with the different HCMV gB genotypes was not significantly different. However, all four patients with infection with a mixture of HCMV gB genotypes developed progression to CMV disease [88]. Coaquette et al. determined the distribution of HCMV gB genotypes and the effect of gB type on clinical outcomes in a cohort of immunocompromised patients, including both transplant recipients and nonrecipients. They found that in contrast to patients infected with a single gB genotype, patients infected with multiple gB genotypes developed progression to CMV disease, had an incresed rate of graft rejection, had higher CMV loads, and were significantly more often infected with other herpesviruses. The presence of multiple gB genotypes, rather than the presence of a single gB genotype, could be a critical factor associated with severe clinical manifestations in immunocompromised patients [89].

Our data clearly indicate the dominance of the gB1 genotype in congenital HCMV infections in south Hungary. However, the clinical outcome of these pregnancies cannot be predicted on the basis of the involvement of this genotype. The diverse clinical findings observed in association with the dominance of the gB1 genotype of HCMV indicate that the gB protein is not the only factor that can influence the virulence of HCMV. The clinical outcomes of intrauterine infections may reflect the contribution of host factors such as immune defense and/or histocompatibility genes. The roles of other viruses (e.g., human herpesvirus 6) or endocrine factors cannot be ruled out.

Previous studies have revealed that a high viral load in the amniotic fluid correlates with CMV disease in the fetus and newborn [90-92]. Revello and coworkers examined pp65 antigenemia, viremia and DNAemia in the peripheral blood leukocytes from newborns born to mother who suffered a primary HCMV infection during pregnancy. Their results indicate that the difference in median viral load in newborns with CMV disease versus those with asymptomatic infection at birth is similar to the difference in viral load observed in the amniotic fluid of a fetus with CMV disease (10^5 GE/ml) versus one with asymptomatic infection [92].

In summary, we were not able to demonstrate significant clinical consequences of HCMV gB genotype and outcomes in congenital HCMV infections. HCMV is a complex virus and its genome contains over 200 open reading frames, including those that code for proteins that help it to evade the immune response and likely many other virulence factors. Other envelope glycoproteins encoded by HCMV such as gH and gCII could influence the importance of gB in virulence and tissue tropism. Further studies into a combination of virulence factors may shed light on the relevance of gB to the development of congenital HCMV infection.

In the matched samples from the mother-infant pairs, the HCMV genotypes were similar. None of the mothers were infected with more than one gB genotype. HCMV strains of different genotypes can be transmitted vertically [80-83]. Thus, control of the passage of HCMV strains across the placenta probably involves a cascade of regulatory events.

Immunological factors

Humoral immune response represents an important defence eliminating HCMV. Therefore it is important to monitorize the level of antibodies in the affected population, especially in pregnant women. It is known that maternal antibodies are not fully protective against HCMV infection but modify the outcome, even the transmission occurs, in those cases when the virus does not cause severe neurological damage. The early determination of maternal antibody status could reveal the affected patients having risk for transmission. The most widely used procedure to screen maternal antibodies is ELISA because of its rapidity, sensitivity and standardized evaluation, although smaller differences can be seen comparing the given results.

A mixture of viral epitopes has been shown to be very efficient in the activation of producing antibodies, but most of them have no neutralizing activity, therefore it is inevitable to investigate the level of neutralizing antibodies. The major target of neutralizing antibodies is the envelope glycoprotein B (gB1-4). The neutralizing antibody titer cannot be evaluated by other serological methods, only by neutralization assay because the correlation of neutralizing antibody titer and the total antibody level is low [93]. At least 30

% of sera with high ELISA titer for the whole virus have little or no neutralizing activity.

Several HCMV strains are circulating in the population at the same time. Measuring the titers of HCMV strain-specific neutralizing antibodies against different strains respectively, a visible difference can be seen. Klein et al. analyzed the extent of HCMV strain-specific neutralization capacity of human sera. Nine HCMV strains were examined and they observed differences from 8-fold to 60-fold in the neutralization titers against each strains. It was concluded that the neutralization capacity of human sera was not influenced by the presence of antibodies against noninfectious envelope proteins and it was independent of the amount of virus [94].

In some cases this can be the explanation of those congenital infections when the maternal immunstatus was positive for HCMV indicating that the mother was infected sometime in the past. However, the mother became infected with another strain of HCMV during pregnancy. The presenting antibodies were not able to neutralize the new HCMV strain therefore the transmission occurred although ELISA showed high level of antibodies.

There are differences in HCMV-specific antibody response between women who transmit the virus to their offspring and those who do not, suggesting that neutralizing antibodies can modulate HCMV spread to endovascular cytotrophoblast (CT) cells. Women with CMV disease, and those with subclinical infection and intrauterine transmission, have a more prolonged and enhanced serum antibody response as compared with those who do not transmit HCMV *in utero* [95, 96]. Women who do or do not transmit HCMV can be differentiated on the basis of their neutralizing antibody titer. Lower titers are present in transmitters versus nontransmitters, suggesting an association between neutralizing antibodies and intrauterine transmission. A further characterization of the antibody responses revealed a marked difference between the two groups of women in the antibody avidity [95]. The avidity of an antibody is indicative of its functional affinity for an antigen. During the first few weeks following a primary infection, the antibodies display a low avidity for the antigen, but as the immune response matures, the antibodies acquire a progressively higher avidity. Both neutralizing antibody levels and avidity develop relatively slowly after a primary infection, suggesting that the mothers who transmit HCMV are in the early stages of primary infection during pregnancy. The failure to produce high-affinity neutralizing antibodies may result in a

greater likelihood of virus dissemination and infection of distant organs, including the placenta. This in turn may increase the probality of fetal transmission.

Our study suggests that gB genotype 1 is the dominant HCMV variant associated with congenital infection. We attempted to find some correlation between the HCMV strain-specific maternal antibodies and the congenital infection and therefore sera of pregnant women and age-matched blood donors as controls were screened in strain-specific ELISAs and neutralization test. The prevalence of Towne and AD169-specific IgG of pregnant women proved to be 82.5% and 78.5%, respectively. Towne-specific IgG was found in 72% of the blood donors only.

According to our results 80% and 83.5% of pregnant women had Towne and AD169-specific neutralizing antibody, respectively. The pregnant women with fetus suspected of having a viral infection had significantly lower specific neutralizing antibody titer to strain Towne comparing to the group with normal pregnancy. The neutralizing capacity to strain Towne in pregnant women, especially pregnant women with fetus suspected of having a viral infection was significantly lower than to AD169. The difference ranged from 4-16-fold in some individuals. Five HCMV infected cases belong to this maternal group, in spite of the high level of AD169-specific neutralizing antibodies. Differences in humoral immunity, and especially the capacity to neutralize strains of HCMV can be one of the reasons why recurrent infection with different strains of HCMV can lead to infection of the fetus in women who are seropositive for HCMV.

This study suggests that the HCMV strain-specific neutralizing antibody present in the maternal blood could inhibit the transmission of the virus to the fetus. The serological determination of strain-specific neutralizing antibodies could be important in judging whether a subject is naturally protected against severe CMV disease. Our data demonstrate differences in the prevalence and capacity of strain-specific neutralizing antibodies in pregnant women and age-matched blood donors, which might have important implication for understanding of the pathogenesis of congenital HCMV infection and development of anti-HCMV vaccines.

The following of our results are considered novel:

1. The gB1 genotype of HCMV predominates in congenital infections in the south of Hungary.

2. The presence of HCMV DNA in amniotic fluid samples from normal pregnancies drew attention to the fact that *in utero* HCMV infection without any negative consequence for the fetus is not a rare event.

3. The clinical outcomes of the pregnancies involving infection with the HCMV gB1 genotype varied. It seems that the gB genotyping of HCMV involved in congenital infections cannot be used to predict the clinical outcome of the infection.

4. Only one gB genotype of HCMV can be detected in all specimens from the mother-infant pair and the placenta.

5. Differences in the prevalence of HCMV strain-specific IgG and neutralizing antibodies can be demonstrated in pregnant women.

6. The prevalence and the neutralizing capacity of the antibodies against different strains of HCMV can reveal differences which can explain why recurrent infection with a different strain of HCMV can lead to infection of the fetus in women who are seropositive for HCMV.

6. Acknowledgments

I express my deepest thanks to my supervisor, Professor Rozália Pusztai for her invaluable scientific guidance and encouragement.

I am particularly grateful to Dr. Emőke Endreffy and Dr. Béla Taródi from professional aspects, it was a great pleasure to work under their skillful guidance and tireless support.

I would like to thank to Professor Éva Gönczöl, the Director of the Department of Medical Microbiology for accepting me as PhD-student and providing the coating antigen for Towne-specific ELISA.

I am grateful to Professor Attila Pál, the Director of the Department of Gynecology and Obstetrics for providing the clinical specimens.

I would like to thank to Dr. Ursula Meyer-König (University of Freiburg, Germany) for providing me the possibility to spend five weeks in her laboratory and to study new molecular methods of HCMV research.

I thank Mrs. Tilda Lévai, Ms. Katalin Hegedűs and Ms. Anikó Salaki for their advice on methodology and the excellent technical assistance.

I would like to thank to Ervin Pörzsölt for helping me in the work on computer.

This study was supported by grants from the Council of Medical Science of the Hungarian Ministry of Welfare (ETT 651/1996), the National Scientific Research Fund (OTKA T-26442/1998) and the Higher Education Research and Development Fund (FKFP-113/2000).

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